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Advanced glycation products' levels and mechanical properties of vaginal tissue in pregnancy

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Running title: Vaginal wall glycation and mechanics in pregnancy

ABSTRACT

Objectives: Non-enzymatic glycation is closely associated with altered mechanical properties of connective tissue. Pregnancy, marked with high levels of female hormones, confers unique alteration to the mechanical properties of pelvic connective tissues in order to meet their physiological demands. However, there are few studies on glycation content and its influence on the mechanical properties of pelvic connective tissues during pregnancy. We hypothesise that the glycation content in pelvic tissues will change with a corresponding change in their mechanical properties, and that these changes are influenced by hormone levels. This study aims to investigate the correlation of vaginal tissue glycation content and mechanical property changes during pregnancy in association with the expression of a key pregnancy hormone (oestrogen) receptor, and an antioxidant enzyme, glyoxalase I.

Study design: A rat vaginal tissue model (tissues from non-pregnant and E15-E18 (last trimester) pregnant rats) was used in this study. Mechanical characteristics of vaginal tissues were analysed by a ball-indentation technique while modulus and morphology of the collagen fibrils within the tissues were measured with atomic force microscopy. A glycation marker, pentosidine, was quantified by a high performance liquid chromatography. The expression of oestrogen and glyoxalase I in the tissue was qualified by immunochemical staining. The glycosaminoglycan (GAG) concentration difference in the tissues were quantified by a biochemical assay.

Results: Pregnant rat vaginal tissue was characterised by significantly lower amounts of pentosidine, higher oestrogen and glyoxalase I expression with larger creep, lower elastic modulus, thicker fibril morphology and higher GAG content than their non-pregnant counterpart. There was a negative correlation between pentosidine and vaginal tissue creep.

Conclusion: There was a reduction in vaginal tissue pentosidine in pregnancy with an associated increase in oestrogen receptor and glyoxalase I immunoexpression. Reduced glycation was associated with increased creeping of vaginal tissue. Oestrogen may therefore play a role in the increase of the vaginal wall's capacity to stretch through glyoxalase I up-regulation and subsequent glycation reduction. The new insight of the correlation of women's oestrogen level, glycation reaction and pelvic tissue mechanical property from this study may enhance our understanding on some pelvic organ diseases.

Key words: pregnancy, mechanics, oestrogen, glyoxalase I, advanced glycation products

INTRODUCTION

Pregnancy alters vaginal tissue mechanics in preparation for delivery (1,2). The vaginal wall is adapted to accommodate delivery of the baby with minimal injury (1,3) and becomes more distensible with a significant decrease in stiffness and ultimate load, returning to its pre-delivery state by the end of the puerperium (1,2,4). Elevation of certain hormone levels is the hallmark of pregnancy. Oestrogen is a notable pregnancy related hormone involved in the remodelling of pelvic connective tissues. It influences collagen metabolism in pelvic connective tissues, causing protein breakdown and neo-synthesis evidenced by an increase in immature collagen crosslinks and decrease in mature crosslinks with collagen therapy (5). Oestrogen therapy has resulted in improved fibroblast mitotic activity, increased turnover of structural proteins like collagen and increased activity of tissue inhibitors of matrix metalloproteinase (6,7). These actions on collagen may influence the proportion of old and new collagen within tissue, thereby altering their mechanical properties.

Older connective tissues contain glycated proteins, nuclei and fatty acids formed by prolonged contact of reducing sugars with susceptible amino acids (8). Glycation occurs by a mailliard reaction in which reducing sugars covalently bind to amino acids. Intermediate products of oxidation formed are rearranged over months or years into advanced glycation end products (AGEs). Within tissues, this process is non-enzymatically regulated. Collagens are prime targets for the glycation process because

of their long half life which leads to prolonged contact with reducing sugars. They also contain significant amounts of susceptible lysine and arginine residues along their chains (9–11).

It is well known that ageing is associated with increased tissue glycation where it alters the mechanical properties of the connective tissues, resulting in stiffer tissues (12). Age-related pelvic floor disorders in women such as pelvic organ prolapse and urinary incontinence are more common after the menopause when oestrogen levels are low. Since oestrogen may influence amounts of old and new collagen in pelvic tissues, and older connective tissue contains more glycation products, oestrogen therefore has the potential to affect the mechanical properties of the tissue by altering glycation content. It is, therefore, hypothesized that there is a relationship between hormonal level, glycation content and mechanical properties of pelvic tissues. Currently few studies correlate hormone level and glycation content in pregnancy. In this study, we aimed to correlate vaginal wall mechanical properties with glycation content during pregnancy by utilising multiple new approaches in a rat tissue model.

MATERIALS AND METHODS

Species and tissue samples

Outbred 6-8 month old female Sprague Dawley rats were used following ethical approval by the Animal Welfare & Ethical Body Review, in accordance with the Animal act of 2006. Full thickness vaginal tissues were obtained from these non-pregnant and E15-E18 (last trimester) pregnant rats post

humane with culling by a Schedule 1 method. Both fresh and defrosted tissues frozen at -20°C were used after the Schedule 1 killing.

Modulus and creep testing

The 24 hour creep deformation and modulus of the intact vaginal tissue segments were measured using a non-destructive ball indentation technique following a pre-established protocol (13). Briefly, the mechanical test device consisted of two transparent plastic O-rings held securely in place by stainless steel screwed plates. Whole intact tubular vagina from the rats were dissected by a mid-line incision and opened up into a rectangular flat piece prior to mounting between the O-rings. Distension of tissues were achieved by central displacement, produced by spherical Teflon or stainless steel balls of weights 0.027 g and 0.27 g for creep and modulus testing respectively. The deformation images were acquired using a microscope with a long focal distance connected to a CCD camera and computer. To obtain creep data, deformation images at time 0 and 24 hour were taken with the tissues incubated in a moist chamber at 37°C and 5% CO₂. The following equation based on the initial displacement, δ , was used for elastic modulus, E, measurement (13):

$$6w/EhR = 0.075(\delta/R)^2 + 0.78(\delta/R)$$

where 'h' represents the tissue's thickness, measured by optical coherence tomography, 'R' is the radius of the spherical ball (4 mm) and 'w' is the weight of the ball.

Ageing biomarker detection

The glycation product, pentosidine, which is formed by the reaction of arginine and lysine with a pentose sugar (8) was chosen for detection (14). Freshly dissected vaginal tissue portions from aged-matched pregnant and non-pregnant rats were hydrolysed in 6 M hydrochloric acid at 110°C over 20 hours. Hydrolysed samples were redissolved in deionised water and separated using high performance liquid chromatography (HPLC, Agilent 1220 LC, USA). Separation occurred in a C-18 250 x 4.60 analytical column and detection at 325 nm wavelength by an ultraviolet detector. Pure pentosidine compound (Caymann Chemical, USA) was used for standard quantification. Pentosidine eluted at 9.6 minutes in a 30 minute run using gradient ratios of 0.1% trifluoroacetic acid and 80% acetonitrile (solvents A and B, respectively) at a flow rate of 1 ml/min with gradient of 0-18% from 0 to 12 minutes and 100% for 12 to 18 minutes for solution B and 100% - 82% from 1 to 12 minutes of solution A.

Immunohistochemistry

Tissues were fixed using 4% paraformaldehyde, dehydrated and processed into wax blocks prior to sectioning into 6 µm-thick sections in a cryostat and immunostained using conventional immunocytochemistry assay. For oestrogen (ER-α) receptor detection, sections were permeabilised with triton-X after blocking by bovine serum albumin. Following incubation with mouse monoclonal IgG antibody to ER-α (Santa Cruz Biotechnology) directed against the intranuclear steroid receptors,

goat anti-mouse IgG (Life Technologies) was incubated against the primary antibody. Rabbit polyclonal glyoxalase I antibody (Abcam, UK) was used to detect its expression with mouse anti-rabbit IgG as the secondary antibody. Nuclei were counter-stained by 4',6-diamidino-2-phenylindole (DAPI).

Nanomechanical properties and Collagen fibril diameter

Atomic force microscopy (AFM) was conducted using a Bruker Multimode instrument (Nanoscope VIII MultiMode AFM, Bruker Nano Inc., CA) equipped with a 150 x 150 x 5 μ m scanner (J-scanner) operated with PeakForce Quantitative Nanomechanical Mapping (PFQNM) modality. For PFQNM measurements, the spring constant of the cantilever and deflection sensitivity were measured on a clean sapphire sample and then, the set up was calibrated with a polymer of known elastic modulus (PSI, Vishay Measurements Group, UK) (15). Five μ m thick vaginal cryosections of pregnant and non-pregnant rats without fixation were collected onto coverslips and stored at -20⁰C until testing. The coverslips were glued to metal support stubs prior to testing. To obtain high resolution images air dried sections were used. A silicon nitride tip (Bruker TAP150) with a nominal tip radius of 8 nm and a 5 N/m spring constant was used for imaging. The elastic modulus for each imaged region of the samples was extracted using the Derjaguin–Muller–Toporov (DMT) model (16). Using ImageJ software, collagen fibril thickness was measured for 45 fibrils per group.

Glycosaminoglycan and water content

Lyophilized weighed rat vaginal tissues were digested in papain enzyme solution (50 ml of 200 mM phosphate buffer containing 1 mM EDTA (pH 6.8), 25 mg papain from papaya latex and 48 mg N-acetylcysteine (17)). Three hundred μ l of digestion solution was added to 6-12 mg dried tissue samples and incubated for 16 h at 60°C. Thereafter, 200 μ l dimethylmethylene blue (DMMB) solution was added to each well of 96 well plate containing 50 μ l of samples or standard solution aliquots, followed by immediate absorbance reading at 530 nm (BioTec Synergy 2). The standard curve was made by using serial dilutions of bovine chondroitin sulphate stock solution. Tissue segments ranging from 4-15 mg were dried to constant weight. Water content of the tissues was determined as the difference between wet and dry tissue weights and expressed as a percentage of the wet weights.

Statistics

An unpaired t-test was performed for comparison of means between pregnant and non-pregnant groups, after determination of data normality using D'Agostino and Pearson omnibus normality tests. A p-value below 0.05 indicated statistical significance. In our graphs, statistical significance is indicated at three levels: $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$ respectively. Standard error of the mean were used to measure variability. Two individual tissue sections per group were studied for collagen fibril modulus and diameter (AFM). Other quantified measurements were based on 5 or more pairs of vaginal tissue.

RESULTS

Using the indentation ball technique, tissue was deformed over 24 hours with a constant weight. Significantly higher deformation was observed in tissue from pregnant rats ($p=0.003$) as compared with non-pregnant rats (Figure 1a). Approximately five times more deformation and 200% lower apparent elastic modulus were observed for the pregnant rat vaginal tissue compared with non-pregnant rats (Figure 1b). These tissue-level mechanical properties were consistent with nanoscale elastic modulus measurements obtained with AFM, showing a trend towards a higher elastic modulus in non-pregnant rats (Figure 2a). Moreover, the nano-scale measurements showed distinct differences between the two groups (Figure 2b). Fibrils within the pregnant tissue appeared wider and merged as shown in the 2D and '3D' images (Figure 3a), they also exhibited more frequent banding, in contrast to the distinctly separated fibrils of the non-pregnant group. Fibril diameter in the pregnant group was significantly greater than the non-pregnant group (Figure 3b). Fibrils within the pregnant group appeared flatter and often merged to form wider 'fibers' unlike the fibrils in the non-pregnant group which had more separated individual collagen fibers.

DMMB assay revealed that GAG content was significantly higher in pregnant rat group ($p=0.02$) than in the non-pregnant (Figure 4a) group with corresponding significantly higher water content in the tissues (Figure 4b).

Pentosidine contents per gram of dry tissue for both groups are shown in Figure 5a. Vaginal tissues from pregnant rats contained an average of 0.02 mg pentosidine per gram of tissue while tissues from

non-pregnant rats contained 0.04 mg pentosidine per gram of tissue. These differences were found to be significant ($P = 0.04$). Good negative correlation ($r^2 = -0.50$) was noted between 24 hour creep and tissue pentosidine content of the same rats (Figure 5 b).

Oestrogen receptor and anti-oxidant enzyme, glyoxalase I expression in both tissue groups were determined by immunostaining assays as shown in Figures 6a-b. Tissue sections from both groups were well nucleated as shown by DAPI staining whilst the intranuclear oestrogen receptors were more strongly expressed in the pregnant than the non-pregnant tissue. Location of glyoxalase I enzyme was predominantly sub-epithelial and more expressed in the pregnant group.

DISCUSSION

Studies on pregnancy induced changes in the latter 2 trimesters of pregnancy have mostly focused on cervical changes rather than the vaginal wall alteration. This study reports for the first time that a glycation product, pentosidine, was significantly reduced during pregnancy, a high oestrogen state; and that this reduction in glycation correlated with higher mechanical creep. Vaginal tissues in pregnancy also expressed higher levels of the antioxidant enzyme, glyoxalase I, than did tissues from the non-pregnant rats. The higher creep behaviour of whole vaginal tissue segments in pregnant rats correlated inversely with their modulus and glycation content. Nano-scale examination of the collagen fibers in pregnant tissue by AFM supported the bulk mechanics change. The morphology and chemical composition changes were observed as significantly thicker collagen fibril diameter and higher GAG

content in pregnant tissues. The regulatory relationship between oestrogen and glycation content in pregnancy was evidenced in this study.

Advanced glycation end products (AGEs) accumulate in tissues, typically increasing with age and causing connective tissue stiffening (14). Although AGEs are associated with many pathologic states, their accumulation within tissues is not always permanent. Macrophages within the body may bind to and scavenge AGEs (18). The high oestrogen state in pregnancy associated with reduced AGEs in the vaginal wall is congruent with other research. Previous findings have noted a decrease in skin autofluorescence (a reflection of advanced glycation) during pregnancy (19). A 50% reduction of pentosidine has also been observed in blood vessels under chronic oestradiol therapy (20). Oestrogen treatment of vascular endothelial cells reduced advanced glycation-induced inflammation (21). This suggests on-going physiologic changes in pregnancy such as modification of connective tissue by pregnancy-related hormones. Pregnancy, or states of high oestrogen expression may therefore involve mechanisms resulting in AGE cleavage and reduced formation. Relationships between glycation products and stiffening of other body tissues such as tendon, intervertebral discs, skin and arteries have been studied but for the first time a relationship is demonstrated in vaginal tissues during pregnancy (22–24). Other studies have similarly reported the association of high glycation with tissue stiffness (22,25).

By a constant load procedure a significantly higher creep and lower stiffness of pregnant vaginas was observed in this study and is congruent with previous studies. The fibromuscular vaginal wall has previously been noted to increase in circumference during pregnancy with apparent collagen reduction (4). The vaginal wall and supporting tissues are more distensible during pregnancy, with a decrease in stiffness, or modulus, in preparation for delivery with minimal injury (1,2,4). Collagen fibrils within the vaginal wall also undergo progressive disorganisation and remodelling through the middle and later stages of pregnancy (26). Although there was apparent thickening of the collagen fibrils in this study, other research suggests that individual fibril diameter may not account for overall tissue modulus (27). Higher tissue modulus typically is the result of crosslinking between fibrils with both enzymatic and non-enzymatic (glycation) crosslinks and is reflected in increased stiffness of the collagen fibers and tissues (8). The significance of reduced glycation crosslinks observed in pregnant tissues may therefore be correlated with altered mechanical properties of the tissues - reduced stiffness and enhanced creep.

Crosslink changes are unlikely to account for the increased collagen fibril diameter observed in pregnant tissues in our study, leading to consideration of another possible mechanism for the observed wider fibrils. Type I collagen fibrils could increase diameter by lateral fusion of fibrils, possibly aiding fiber performance at high strain (28). Our AFM images revealed collagen fibrils which appeared fused to form the wider fibrils. To further understand the observation, GAGs contents were measured and were notably higher in pregnant rat tissues. Previous studies have also noted a progressive increase in

GAGs content of pelvic tissues during pregnancy and its loss at delivery (26,29). Certain proteoglycans also modulate fibrillogenesis and fiber alignment (30). Studies have shown that removal of GAGs results in disorganization of collagen fibrils (31). GAGs present between collagen fibrils in connective tissues may aid their arrangement, resisting complete fiber disorganisation during stretching. GAGs influence collagen fibril association through methods other than direct chemical or physical crosslinking (32), causing an apparent increased fiber diameter and resisting complete deformation during sliding. Thus, the higher expression of GAG in pregnancy may serve as a protective measure during tissue stretching by preventing complete disorganisation of fibrils.

In pregnancy, oestrogen may influence tissue glycation by modulating the oxidative stress pathway. Glycation is associated with oxidative stress and reactive oxygen species have been shown to trigger AGE formation (33,34). Excessive production of reactive oxygen species within the mitochondria of cells inhibits glyceraldehyde-3-phosphate dehydrogenase and diverts substrates from glucose breakdown pathways to increased AGE formation (35). Oestrogen administration has been associated with reduced oxidative damage and glycation induced stress within connective tissues (20,36). Oestrogens cause increased expression and activity of antioxidant enzymes and their co-factors (37). Reduced oestrogen is associated with decreased amounts of the thiol antioxidant, glutathione (GSH) which is reversed by oestrogen administration (37). Oestrogen also influences the glutathione-dependent glyoxalase system through glyoxalase I which plays a role in the removal and prevention of

AGEs accumulation by detoxification of methylglyoxal, a reactive dicarbonyl intermediate and AGE precursor (34,38). Deficiency in glyoxalase I enzyme has been implicated in increased AGE accumulation within tissues (39). In our study, we noted increased expression of glyoxalase I enzyme in the tissues from pregnant rats similar to findings in other studies (40). A high oestrogen level is also associated with increased activity of glutathione peroxidase, a potent antioxidant (41). This would lead to more removal of reactive oxygen species and subsequent reduced glycation. Thus, in addition to on-going physiologic removal of AGE by macrophages within tissues (42,43), a decrease in glycation of tissues resulting in reduced formation of AGEs is expected. Hence states of higher oestrogen such as pregnancy or the proliferative phase of the female menstrual cycle would be associated with reduced AGEs through a proposed oestrogen-gluthathione-glyoxalase pathway as depicted in Figure 7. As a result, whilst oxidative stress promotes AGEs formation, oestrogen increases the activity of glyoxalase and GSH leading to stronger detoxification, production of lactoylglutathione and decreased AGEs. This physiologic pathway would also be useful in understanding the pathogenesis of pelvic organ prolapse which occurs predominantly in older menopausal women. During the years following menopause oestrogen levels decline and this may halt the process of cyclical removal of AGEs which should occur during the normal reproductive life of the female. Progesterone is another significant hormone during pregnancy. However there is minimal evidence for its influence on AGEs. Further

investigation on the effects of progesterone on glycation may however be useful in obtaining a more robust understanding of the effect of pregnancy on AGEs in pelvic tissues.

This study used a minimally-destructive technique for assessment of vaginal tissue bulk mechanical property and employed the AFM technique for the study of collagen fiber mechanics and morphology, enabling simultaneous assessment of the global and nano-scale mechanical and structural properties of vaginal tissues. The limitation of this study is that it is based on an animal model. It is desirable to study human samples, both vaginal and hormonal, to confirm the observations in this study.

COMMENTS

This study is the first of its kind and highlights a potential relationship between mechanical changes observed in pregnancy and collagen fiber changes within the connective tissue, confirming that pregnant vaginal tissues contain lower levels of advanced glycation end products. Glycation reduction may contribute to the increased flexibility (high creep) of pregnant vaginal tissues in association with increased GAG levels and collagen fiber fusion. Pregnancy reduced vaginal wall glycation by increased levels of the antioxidant enzyme glyoxalase I, and oestrogen, is potentially a major reason for the observed changes. These observations generate novel insights into other pathways for vaginal tissue mechanical changes during pregnancy and provide ~~some~~ evidence that the alteration of glyoxalase concentration by the elevation of oestrogen levels correlates with a reduction in AGEs thus altering vaginal tissue flexibility.

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DISCLOSURES

There are no conflicts of interests.

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FIGURE CAPTIONS

Figure 1 Mechanical properties of intact vaginal tissue segments from pregnant and non-pregnant rats measured by the ball indentation technique. (a) 24 hour creep (* $p=0.003$); (b) Elastic modulus (* $p=0.047$).

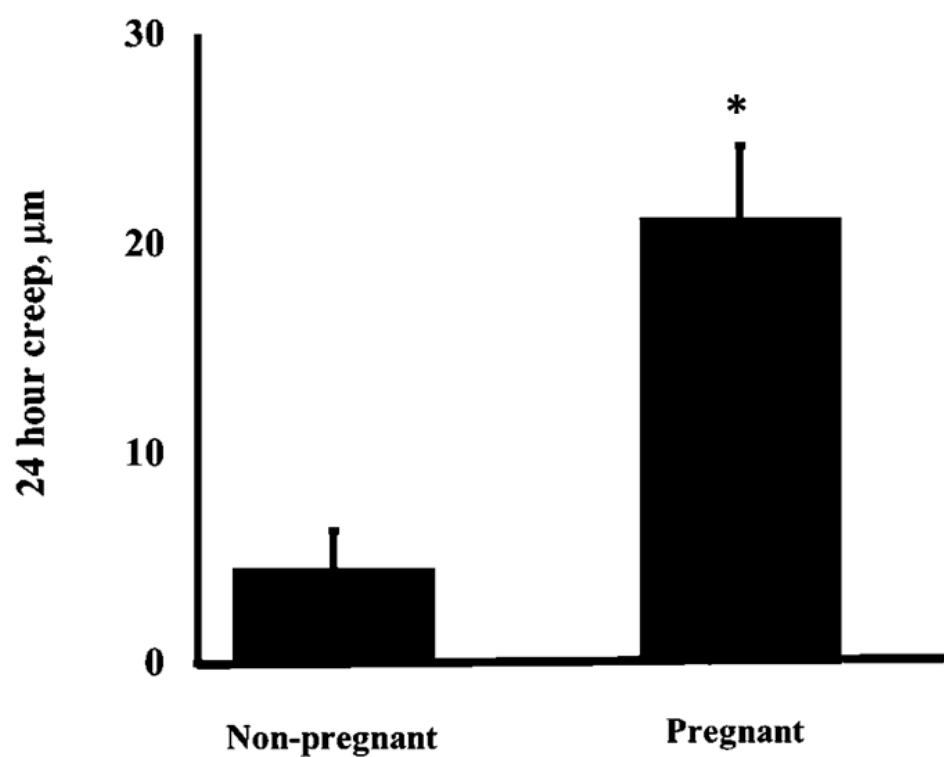


Figure 1a

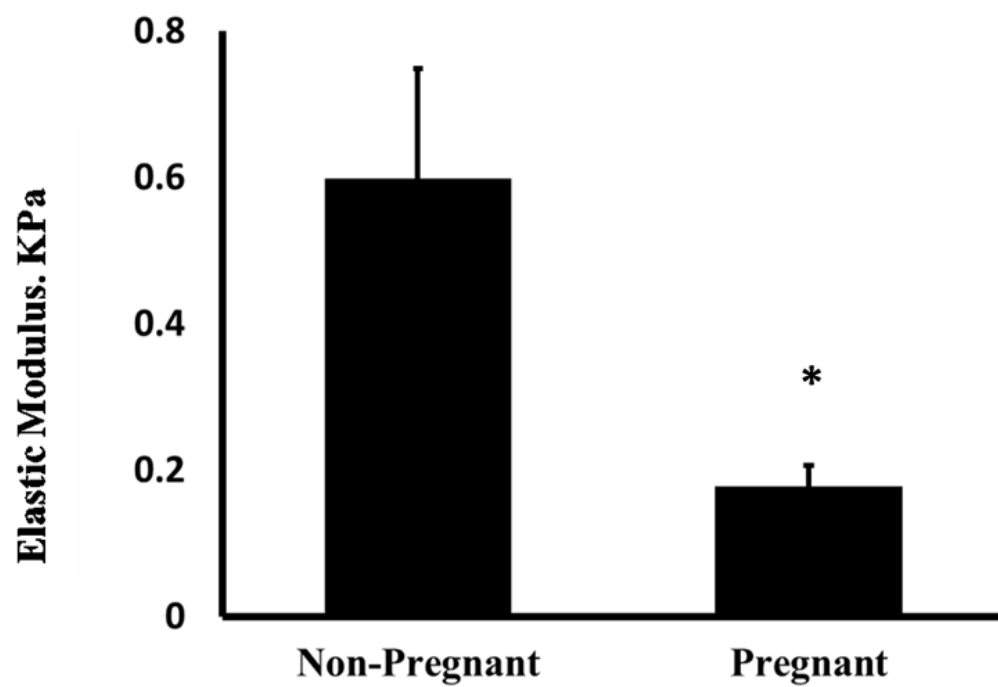


Figure 1b

Figure 2 AFM measurement of mechanical property. (a) Representative maps showing modulus range over a $1.4\ \mu\text{m}^2$ area of vaginal tissue sections from pregnant (left) and non-pregnant (right) rats. The color bars alongside the images indicate the modulus value; (b) Mean modulus of collagen fibrils in the both tissues ($p=0.335$).

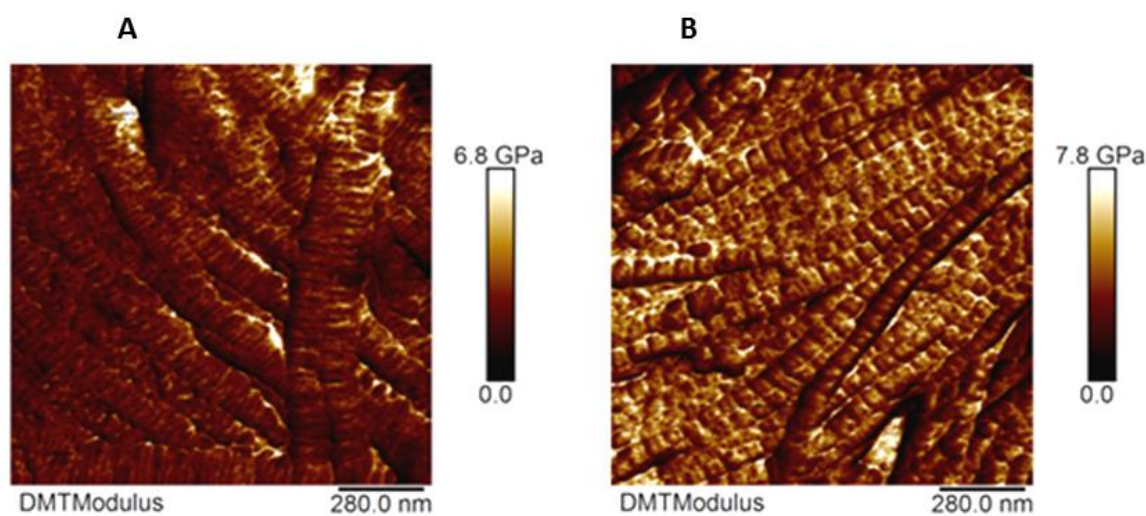


Figure 2a

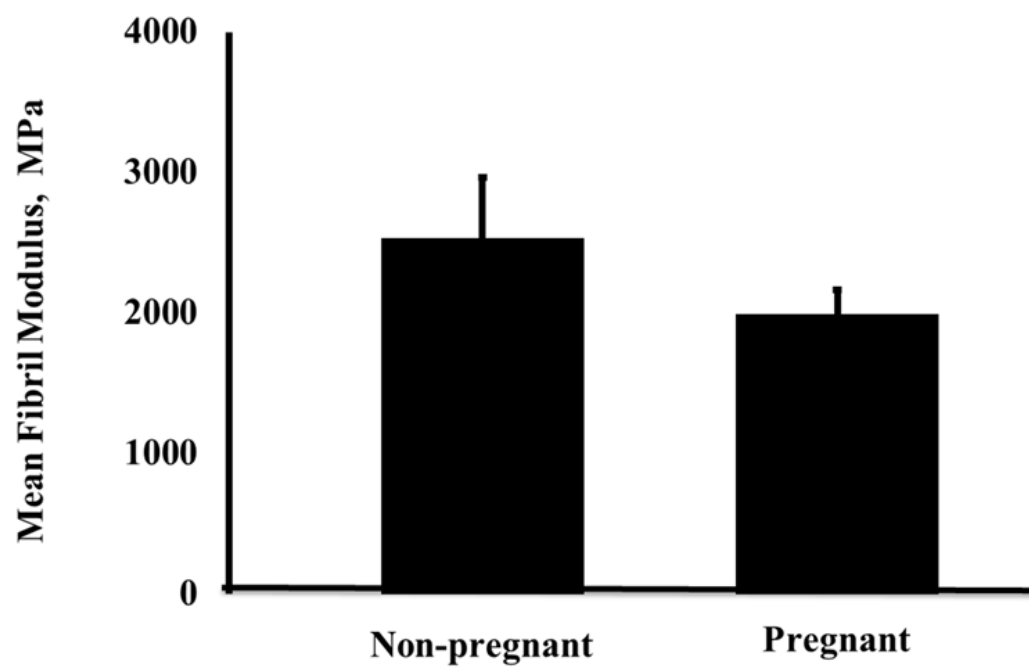


Figure 2b

Figure 3 AFM measurement of collagen fibril morphology of pregnant and non-pregnant vaginal tissues. (a) Representative 2-dimensional (A and B) and 3-dimensional (C and D) images of collagen fibrils; (b) The mean diameters of collagen fibrils (***) $p=0.000$. 15 collagen fibrils from $3 \times 1.4 \mu\text{m}^2$ regions of $3 \times 5 \mu\text{m}$ thick sections have been analysed. Arrows indicate merging of the fibrils.

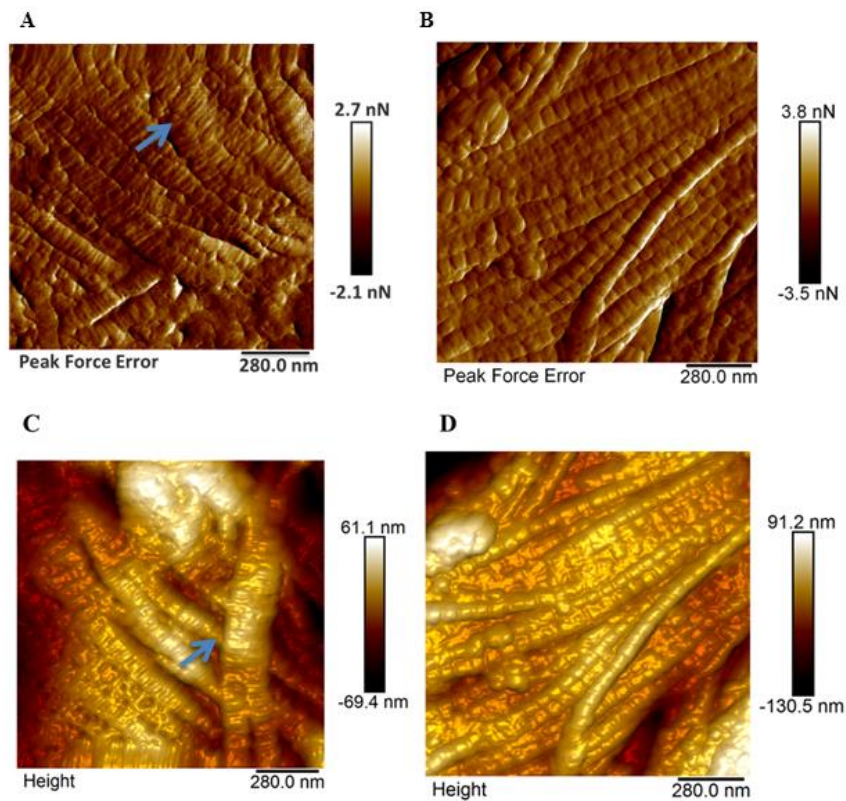


Figure 3a

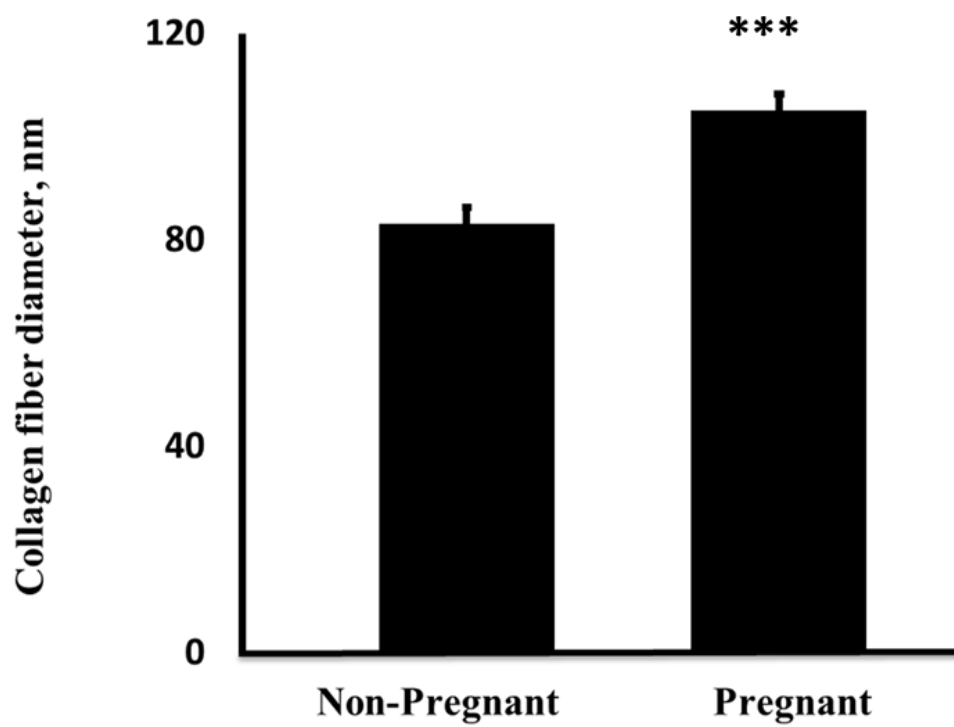


Figure 3b

Figure 4 (a) GAG content per dry weight of pregnant and non-pregnant rat vaginal tissues (* p value=0.015); (b) Percentage water content of pregnant and non-pregnant rat vaginal tissues (* p value=0.047)

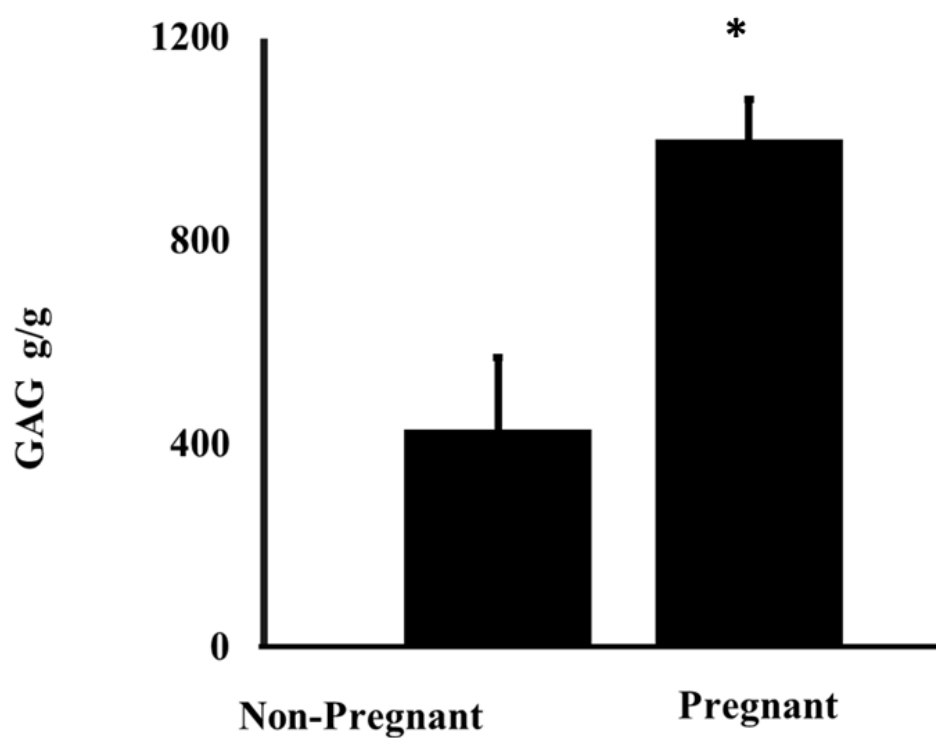


Figure 4a

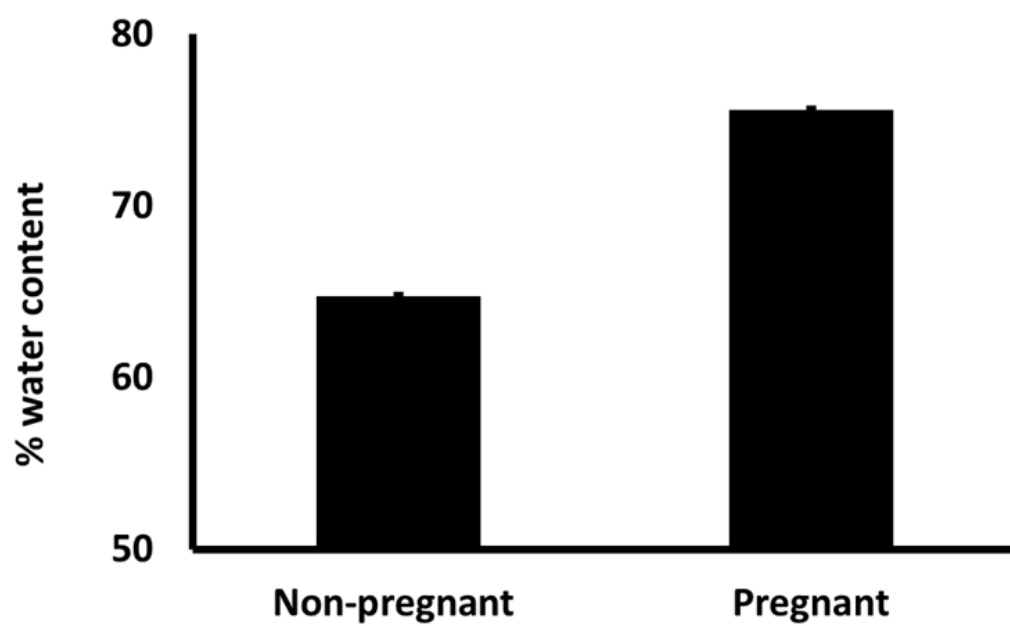


Figure 4b

Figure 5 (a) AGE marker level measured from HPLC for the pentosidine content in vaginal tissues from pregnant and non-pregnant rats (* p value=0.042); (b) Correlation between pentosidine and vaginal tissue creep ($r^2 = -0.50$)

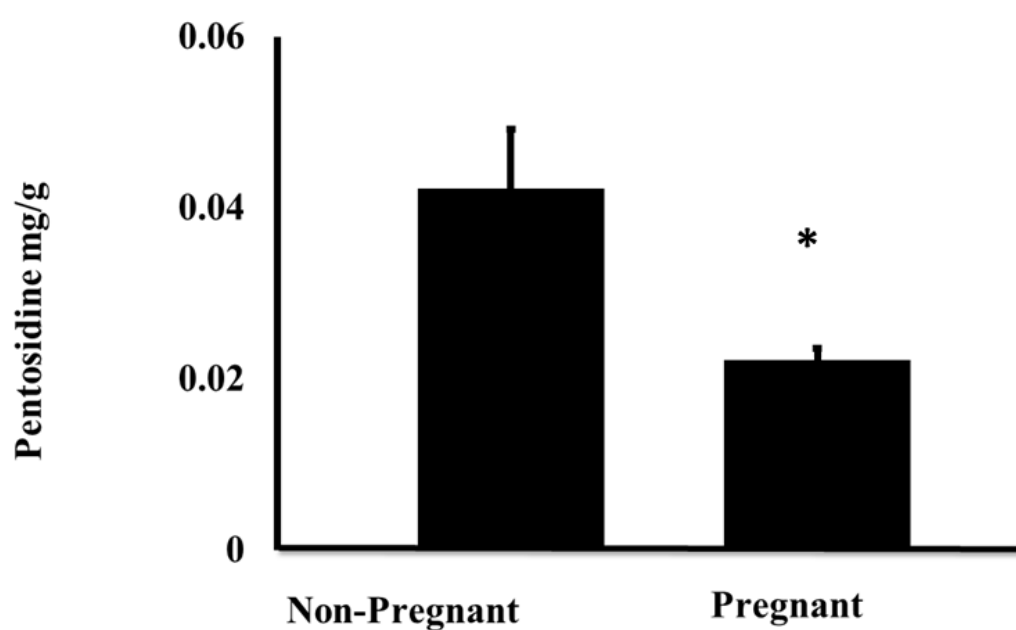


Figure 5a

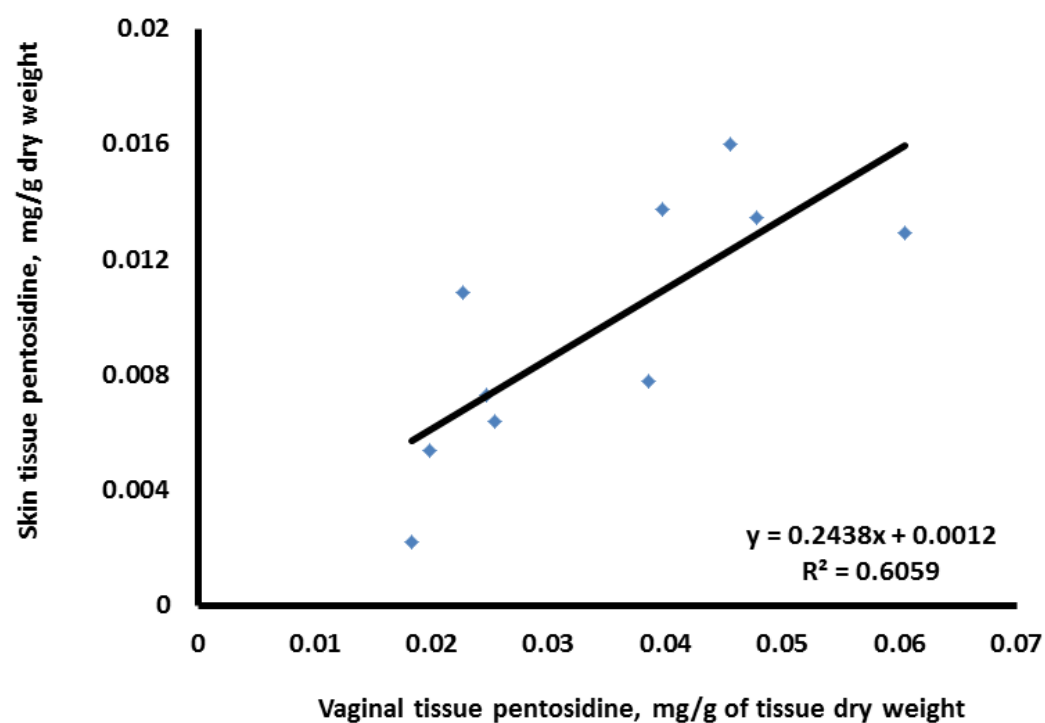


Figure 5b

Figure 6 Immunofluorescence staining images of vaginal tissue sections. (a) Representative images of ER- α receptor expression; (b) Representative images of glyoxalase I expression. P: pregnant; NP: non-pregnant; Tritc: ER- α receptor; FITC: glyoxalase-1; BF: bright field and Dapi: nuclei. Scale bars = 50 μ m.

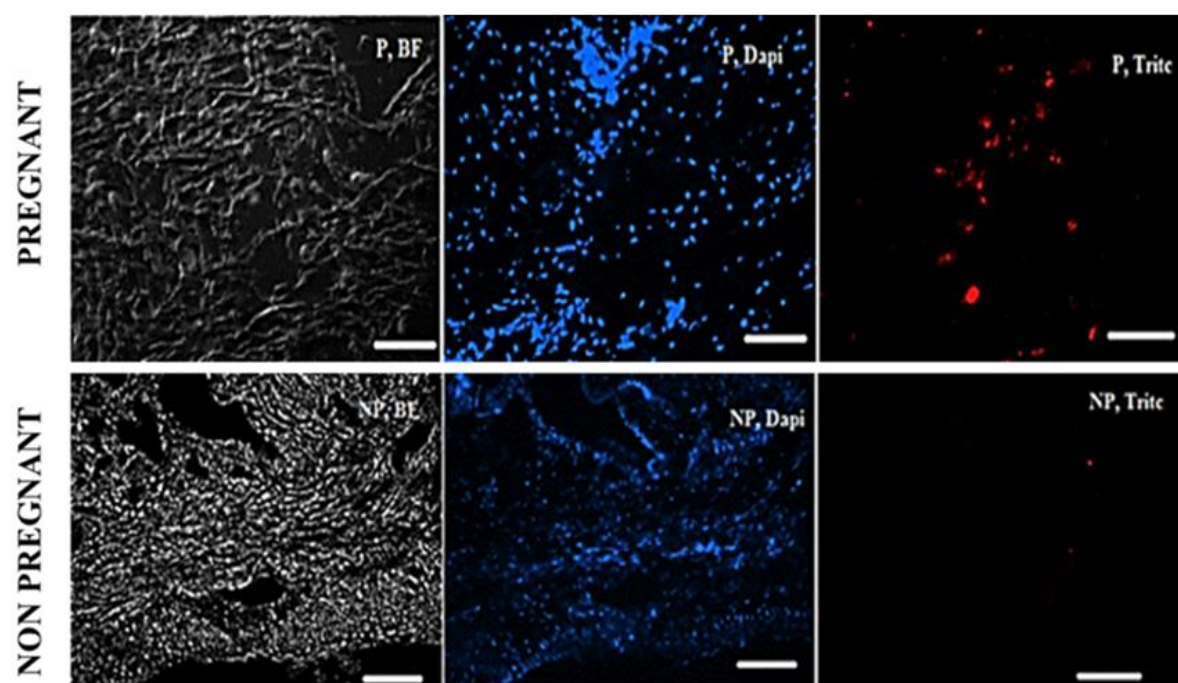


Figure 6a

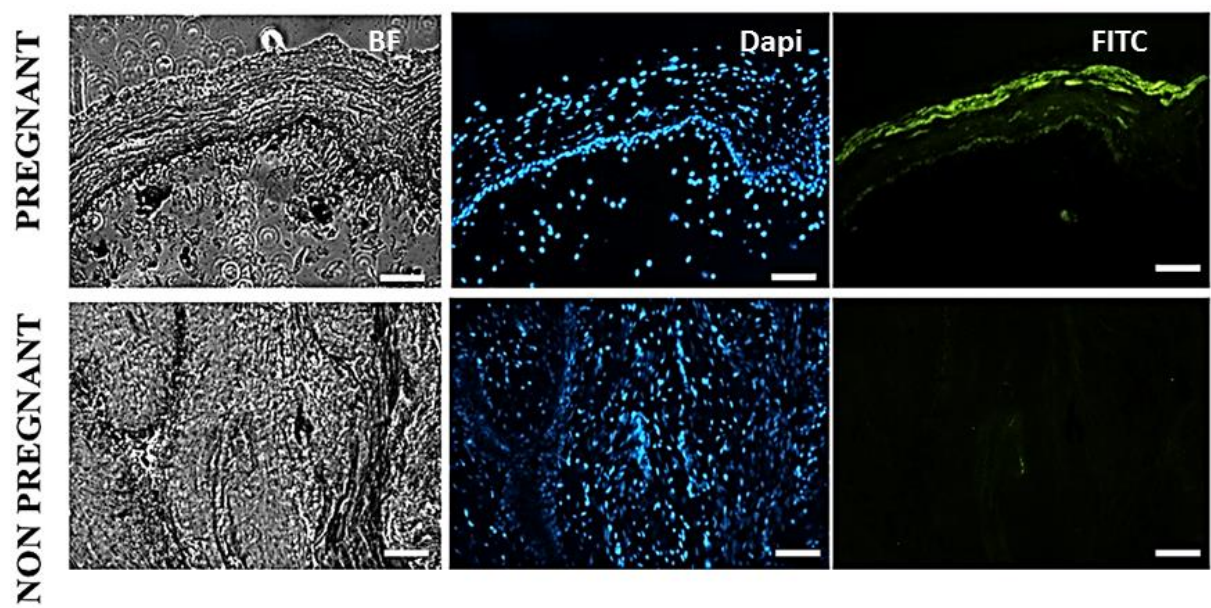


Figure 6b

Figure 7 Illustration of AGEs formation inhibition by oestrogen. Oestrogen increases antioxidant enzyme and co-factor, glyoxalase I and GSH. In the detoxification processes, intermediate AGE precursors, e.g. methylglyoxal, are diverted to other pathways, leading to reduced AGE accumulation.

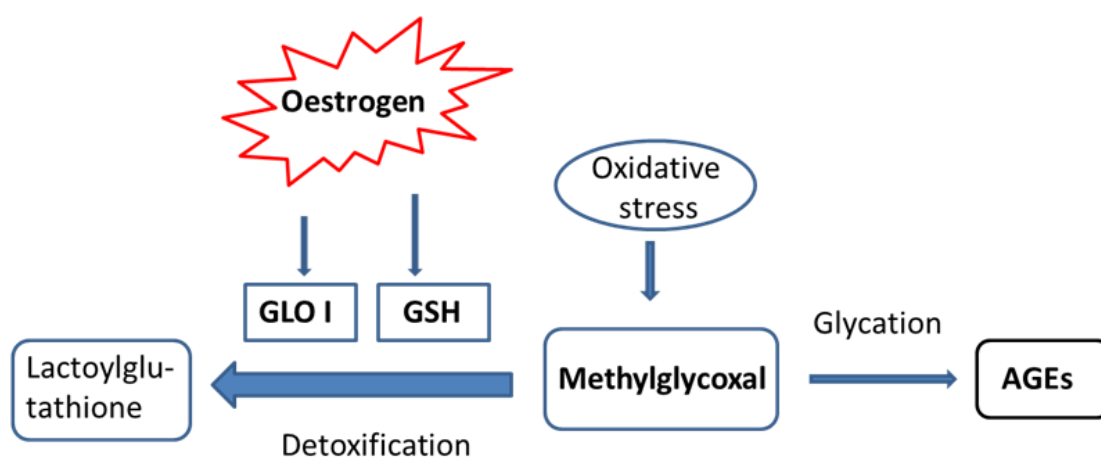


Figure 7